

Evaluation of transgenic *Prunus domestica* L., clone C5 resistance to *Plum pox virus**

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Abstract

Plum pox virus (PPV) is one of the most devastating diseases of *Prunus* species. Since few sources of resistance to PPV have been identified, transgene-based resistance offers a complementary approach to developing PPV-resistant stone fruit cultivars. C5, a transgenic clone of *Prunus domestica* L., containing the PPV coat protein (CP) gene, has been described as highly resistant to PPV in greenhouse tests, displaying characteristics typical of post-transcriptional gene silencing (PTGS). Moreover, C5 trees exposed to natural aphid vectors in the field remained uninfected after 4 years while susceptible transgenic and untransformed trees developed severe symptoms within the first year. In our study, a high and permanent infection pressure of PPV was provided by bud grafting of inoculum in the field trial of clone C5 conducted in the Czech Republic, in which PPV-infected and healthy control trees were used. Moreover, trees with combined inoculations by PPV, ACLSV and PDV were also used in the trial. The presence of the viruses throughout the tree tissues, the relative titre of the viruses and symptoms on C5 trees have been monitored over the years. The resistance stability of C5 clones under permanent infection pressure is discussed.

Keywords: PPV, C5, resistance, real-time PCR

Introduction

Plum pox potyvirus (PPV) is the causal agent of 'Sharka' disease in *Prunus* species and is considered to be one of the most serious diseases of stone fruit trees in Europe. PPV has recently been detected in North and South America (Gildow et al., 2004). Following the successful transfer of the PPV coat protein (CP) gene to *Prunus domestica* plum (Scorza et al., 1994), the transgenic plum clone C5 has shown to be highly and steadily resistant to PPV infection (Ravelonandro, 1997). The stability of resistance to PPV has also been proved in long term field experiments in Poland, Spain, Romania (Malinowski et al., 2006) and more recently in the Czech Republic (Polak et al., 2008). The PPV resistance in C5 was previously demonstrated to be associated with post-transcriptional gene silencing (PTGS) (Scorza et al., 2001) and RNA silencing associated short interfering RNA duplex (siRNA: 21-22 nt and 25-26 nt) were detected in C5 plant (Hily et al., 2005). It was also demonstrated that long class siRNAs (25-26 nt) mediate the systemic silencing and play significant role in conferring the resistant phenotype in C5 to PPV infection (Kundu et al., 2008).

Here we have evaluated the field resistance of C5 in a permanent and continual inoculum pressure through challenging the plants with either PPV-Rec solely or in combination of heterologous viruses: *Prune dwarf virus*-PDV and *Apple chlorotic leaf spot virus*-ACLSV by grafting inoculation. The appearance of symptoms was evaluated and the presence of inoculated viruses were tested by RT-PCR using specific primers for PPV-Rec (Subr et al., 2004), PDV and ACLSV (Polak et al., 2008). The PPV concentration in C5 was compared with non-silenced root stock St. Julien by SYBER green based absolute quantification by Real-time RT-PCR.

Material and methods

Virus detection in C5 by RT-PCR: The experiment to evaluate the influence of heterologous viruses: combinations of PPV-Rec together with PDV and ACLSV were graft-inoculated on C5 trees as follows: PPV+PDV, PPV+ACLSV, and PPV+ACLSV+PDV (11 C5 in each combination), and also PPV-Rec as a single infection (9 C5 trees). Leaf samples were collected, grinded in liquid nitrogen and total RNA was isolated by RNeasy Mini Plant extraction kit (Qiagen, USA) with modification as in (Mekuria et al., 2003). One Step RT-PCR was carried out as in (Kundu, 2003). One Step RT-PCR was performed with the One Step RT-PCR kit (Qiagen) as follows: the One Step RT-PCR mixture containing 5 µl of the 5x Qiagen One Step RT-PCR buffer, 10 nM of each dNTP, 1 µl of the Qiagen One Step RT-PCR enzyme mixture, 1 µl of Q solution, 6 pM of upstream and downstream primers were prepared in microtubes and 1 µl of RNA was added. The mixture was adjusted to 25 µl with RNase-free water. The reaction was carried out in a thermocycler (MJ Research) as follows: a RT step at 50°C for 30 min and an initial PCR activation step at 95°C for 15 min, then 33 cycles of 94°C for 30 s (denaturation), 55-60°C for 45 s (annealing), and 72°C for 80 s (extension). After the last cycle, a final extension step at 72°C for 10 min was added. The PCR products were analyzed in 1% agarose gel electrophoresis, staining was done by SYBR Green (Invitrogen, California, USA).

PPV quantification in C5 by Real-time RT-PCR: Nine C5 transgenic plum trees grown in an experimental orchard were bud-grafted with plum cv. 'St Julien' infected with PPV-Rec strain (Polak et al., 2008). The leave samples from the trees were collected during years 2006, 2007 and 2008. Always, three samples were collected per one tree - leaves of the 'St Julien' infectious bud (the non-transgenic part), leaves of bottom part of the C5 (leaves close to the infectious bud, transgenic part) and leaves of top part of the C5 (far from the infectious bud, transgenic part). The material was grinded in liquid nitrogen and several aliquots of 0.1g were stored at -80°C. Thereafter, total RNA extraction was performed by RNeasy Mini Plant extraction kit (Qiagen, USA) with modification as in Mekuria et al., 2003. The primer pair specific for

PPV-Rec targeting (Cter) N1b-(Nter) CP 8532-8669 (NCBI Acc. Number [AY028309](#)) RecJF:
5' AATGATATTGATGATAGCCTTGAC-3' and RecJR 5'-AGCTGGTTGAGTTGTTGCCAC-3'

amplifying a 138bp product. Specificity of the PPV-Rec primers was checked by Real-time RT-PCR on PPV-D, PPV-M and PPV-Rec isolates (data not shown).

The specific PPV-Rec fragment described above was inserted into the vector pGem-T (Promega Inc.) and cloned into *E. coli* JM-109. The plasmid was linearized at the *Rsa* I site and used as target in an in vitro transcription reaction performed with Megascript T7 kit (Ambion Inc., TX) followed by DNase I treatment (DNAfree, Ambion). The amount of RNA was quantified by UV densitometry. Conversion of microgram of single stranded RNA to picomole was performed considering the average molecular weight of a ribonucleotide (340 Da) and the number of bases of the transcript (Nb). The following mathematical formula was applied: pmol of ssRNA = $\mu\text{g (of ssRNA)} \times (106 \text{ pg}/1\mu\text{g}) \times (1 \text{ pmol}/340 \text{ pg}) \times (1/\text{Nb})$. Avogadro constant was used to estimate the number of transcripts (6.023×10^{23} molecules/mol) (Olmos et al., 2005). Subsequently, ten-fold serial dilutions of the transcripts were prepared and used. Real-time RT-PCR was performed with SYBR Green I, using a 7300 Real-time PCR System (Applied Biosystems, CA, USA) and Power SYBR Green RNA-to-CT™ 1-Step Kit (Applied Biosystem, USA) according to the recommendation of the manufacturer.

Results and discussion

Nine C5 plants were inoculated with PPV-Rec. PPV was detected in eight C5 plants, in most of the cases close to the inoculum sources (Table 1). Mild diffuse mosaic symptoms were observed (Figure 1A, B) also in those part of C5 scion. The upper leaves remained symptomless and virus free (except two C5 scions in which PPV were detected by RT-PCR). These results agree with our previous observation that in the permanent inoculum pressure (from non-silenced St. Julien), C5 scions become virus infected and also exhibit symptoms (Kundu et al., 2008). In such circumstances the siRNA mediated silencing system perhaps overwhelmed and blocked the accumulation of long class siRNA (26-27 nt) in C5 (Kundu et al., 2008), which is significant for systemic silencing in plant (Hamilton et al., 2002). ACLSV and PDV viruses were also detected by RT-PCR and results are shown in Table 2. Neither PDV nor ACLSV seemed to have any effect on C5 resistance to PPV infection.

Tab. 1 Number of positives plants during years 2006, 2007 and 2008

	Number of PPV positive/ number of tested plants		
	2006	2007	2008
Leaves of St Julien	7/8	7/8	7/8
Bottom leaves of C5	8/9	8/9	8/9
Top leaves of C5	0/9	2/9	2/9

Tab. 2 Detection of PPV, PDV and ACLSV during the years 2006, 2007 and 2008

		Number of virus positive/ number of tested plants					
		2007			2008		
		PPV	PDV	ACLSV	PPV	PDV	ACLSV
Grafting PPV+PDV	C5	4/11	0/11	xxxx	4/11	0/11	xxxx
Grafting PPV+ACLSV	C5	4/11	xxxx	11/11	4/11	xxxx	11/11
Grafting PPV+PDV+ACLSV	C5	5/11	0/11	11/11	5/11	0/11	11/11

PPV and ACLSV were also detected in the each of individual grafted bud for each virus. PDV grafted bud are missing in the plants

The resistance level of C5 to PPV-Rec was furthermore evaluated by Real-time RT-PCR. The field growing C5 plants inoculated with PPV-Rec (from Table 1) were subjected to our analysis. The results of absolute quantification have shown that the average concentration of PPV-Rec in non-silenced root stock (St. Julien) increased gradually over the tested period compared to C5. In C5, the virus concentration remained more or less similar and was slightly increased in 2008 (see Figure 1). The virus concentration in silenced C5 was significantly lower than in the non-silenced St. Julien

(the permanent inoculum sources) (see Table 3). The low level of PPV concentration has again confirmed the stability of resistance in C5 to a Rec isolate in a high and continuous infection pressure in field conditions.



Fig. 1 PPV symptoms in field. **A:** mild diffuse spots in the leaf of C5 inoculated with PPV-Rec. **B:** very mild diffuse spots in the leaf of C5 inoculated with PPV-Rec. (Fifth year after inoculation, July 2007.) **C:** Severe yellow spots and rings in the leaf growing from non-transgenic bud St. Julien (IB) infected with PPV-Rec.

Tab. 3 PPV copy number in St. Julien versus C5

	Year of the experiment		
	2006	2007	2008
Bottom leaves of C5	~16 fold	~300 fold	~ 11 fold
Top leaves of C5	~7300 fold	~5500 fold	~ 16 fold

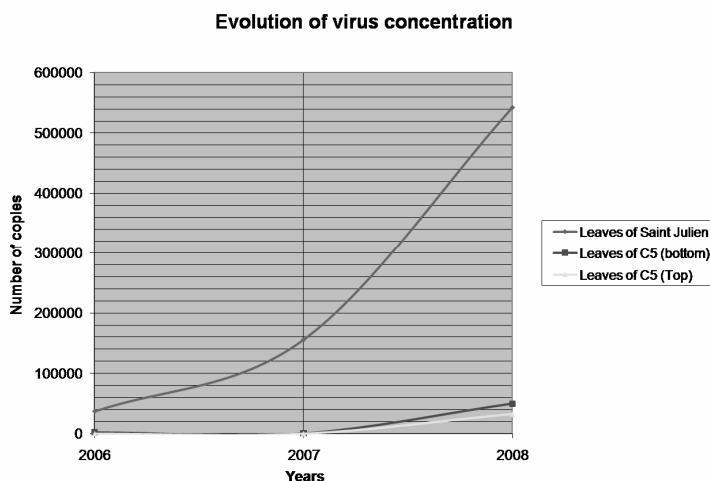


Fig. 2 PPV-rec concentration in C5 plants, detected in 1) PPV-rec inoculum source (St. Julien), 2) Bottom leaves of C5 (close to the inoculum source) and 3) Top leaves of C5. Average of eight individual tree \pm standard error are given.

In conclusion, C5 plants maintained high level of resistance to PPV-Rec over the six years period after graft inoculation by susceptible st. Julian graft in field conditions. The Sharka symptoms appeared in most of the C5 plants, however, only very mild diffuse spots were observed. PPV concentration in C5 plants was significantly lower than in the non-silenced root stock. In the absence of inoculums pressure, the C5 plant became virus free over the time in field conditions probably due to the active systematic silencing. The above mentioned results from the field test and previous results from the green house (Kundu et al., 2008) showed that under a high and continual inoculums pressure, the virus can infect the C5 plant at only a very low level and that the active silencing confers the resistance to PPV in this woody perennial.

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